AN INVESTIGATION OF BACTERIOPHAGE IN CHEESE STARTERS

BY

HOWARD ERRETTE MOORMAN

B.Ed., Southern Illinois University, 1936
M.S., University of Illinois, 1942

AN ABSTRACT OF A THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN DAIRY PRODUCTION
IN THE GRADUATE COLLEGE OF THE
UNIVERSITY OF ILLINOIS, 1949

URBANA, ILLINOIS

1949

AN INVESTIGATION OF BACTERIOPHAGE IN CHEESE STARTERS

BY

HOWARD ERRETTE MOORMAN

B.Ed., Southern Illinois University, 1936
M.S., University of Illinois, 1942

AN ABSTRACT OF A THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN DAIRY PRODUCTION
IN THE GRADUATE COLLEGE OF THE
UNIVERSITY OF ILLINOIS, 1949

URBANA, ILLINOIS

1949

ACKNOWLEDGMENT

The author desires to express his sincere appreciation to Dr. R. V. Hussong for guidance and aid in carrying out this work.

The author also wishes to thank Drs. G. W. Salisbury and F. M. Clark for their kind assistance and helpful suggestions in the preparation of this abstract.

R. UNIV.
BIBLIOTHEEK
LEIDEN

AN INVESTIGATION OF BACTERIOPHAGE IN CHEESE STARTERS

Since the existence of bacteriophage first was postulated by D'Herelle in 1917, much of the investigation has been concerned with the relation of bacteriophage to disease and with its relationship to other living entities. Recently, the phenomenon of bacteriophagia has been related directly to the dairy industry. On numerous occasions it has been demonstrated that bacteriophage was a cause of slow starters, a reduction in the rate of acid production by starter cultures used in the manufacture of cheese. In this activity, bacteriophage varies in its reaction and may merely retard acid development by the starter or it may inhibit, almost completely, the production of acid by the starter cultures. Because of the economic considerations involved by the losses of product, time, and labor, the problem of bacteriophage contamination in the dairy industry is critical.

The objectives of this study were threefold:

1. Methods for detection of *Streptococcus lactis* bacteriophage.
2. Isolation of *S. lactis* bacteriophage from pure cultures.
3. Control of *S. lactis* bacteriophage.

A study was made of the more common methods of detecting phage, measuring and increasing the number of phage particles per ml., methods of filtration, and storage of phage filtrates.

The experimental cultures were commercial cheese starter cultures and were obtained from cheese manufacturers and from Purdue University.

Bacteriophage was isolated from whey samples obtained from the cheese manufacturer which submitted the starter cultures. Bacteriophage strains active against starter cultures received from Purdue University were isolated from whey samples obtained from a central Illinois cheese factory.

Detection

Detection of Bacteriophage. After preliminary experimentation with various tests designed to detect bacteriophage, it was decided to employ a modification of a test used by one of the leading cheese manufacturers. This test consisted in the inoculation of two tubes containing nine ml. of litmus milk with 0.1 ml. of the susceptible starter culture and then inoculating 0.45 ml. of filtered whey from the slow vat into one of the tubes and incubating overnight at 30°C. When the tube into which the test whey was inoculated failed to show acid, reduction, and coagulation comparable to the culture control tube, bacteriophage was suspected.

Measuring and Increasing Titer. The plaque method and the titer method were investigated as a means of determining the number of phage particles per ml. The titer test was adjudged to involve less labor, to be more rapid, and as effective as the plaque test. Consequently, the titer test was selected for the ensuing experimentation.

The maximum increase in titer was obtained by controlling the number of new host cells and by incubation of the medium at the optimum temperature for the phage strain. The number of new host cells was controlled by the amount of inoculum used and by incubation at 30°C. for four hours before inoculation with phage. The optimum temperature of the experimental strains of *S. lactis* phage was found to be 30°C.

Filtration. A comparison was made of the Seitz, Jenkins, and Selas 03 filters. The Seitz filter was found to permit more rapid filtration but produced many more contaminated filtrates than the other two filters being tested. Because the Selas 03 produced a lower number of contaminated filtrates and the time difference was not too great, the Selas 03 was selected for obtaining bacteria-free filtrates.

Filtrate Storage. A comparison of filtrates and neutralized filtrates was made with aliquot portions of the same filtered whey or broth sample. The filtrates were divided into two aliquot portions, one portion neutralized with N/10 NaOH,

and both portions stored at 4°C. for nine months. Periodically, samples of the filtrates were withdrawn and titers determined. It was observed that neutralized filtrates retained a higher titer for a longer period of time than did the non-neutralized filtrates.

Isolation From Pure Cultures

Isolation from Slow Starter Cultures. Starter cultures A, B, and C were plated out on tomato juice agar and incubated at 30°C. for 48 hours. Fifty colonies of each starter were picked to tubes of litmus milk and incubated at room temperature for one to five days until maximum growth had been reached. The cultures were separated into four categories based on the amount of acid, coagulation, and reduction:

Group 1. Reduced-almost entirely coagulated-acid.

Group 2. Reduced-slight coagulation-slight acid.

Group 3. Silght reduction-no coagulation-very slightly acid.

Group 4. No reduction-no coagulation-no acid.

The pooled tubes were filtered and the four bacteria-free filtrates tested for phage activity against strains of the starter from which the filtrates were obtained.

The Low Temperature Method. Numerous cultures which had previously shown slowness in acidification, coagulation, and reduction were inoculated into tubes of litmus milk, protolysate broth, and onto protolysate agar slants. These tubes were incubated at 30°C. until a good growth was visible and then placed in the walk-in ice box at 4°C. After a fourteen day incubation period the tubes were withdrawn and five ml. of distilled water added to the protolysate agar slants. The bacteria-free filtrates obtained by the subsequent filtration were tested for phage activity against the strains from which the filtrates were obtained.

The Lithium Chloride Method. Cultures were inoculated onto nutrient agar slants containing one percent lithium chloride and one percent protolysate. After an incubation period of

seven hours at 30°C. the slants were washed with distilled water, centrifuged at a Relative Centrifugal Force of 13,215 grams, and the supernatant liquid filtered. The bacteria-free filtrates obtained were tested for titer and plaque formation.

Isolation from Casein. Tubes of 2.5 percent casein were adjusted to pH 7.0 with N/10 NaOH and autoclaved at 121°C. for fifteen minutes. The tubes were inoculated heavily with slow starter cultures and incubated at 13°C. for twenty-eight days. At the termination of the incubation period bacteria-free filtrates were prepared from the tubes and tested for titer and plaque formation.

Results — None of the foregoing methods produced filtrates capable of lysing starter cultures.

Conclusion — Although there is a possibility of isolating phage from pure cultures of slow starters, no evidence was forthcoming to indicate that phage was present in the slow starter cultures tested.

Control

In the search for an efficient inhibitory agent for phage it was decided to investigate the possibilities of the quaternary ammonium compounds. Six compounds were selected and each was manufactured by a different company. The compounds selected were:

alkyl dimethyl benzyl ammonium chloride
cetyl pyridinium chloride
lauryl iso quinolinum bromide
diiso butyl phenoxy ethoxy ethyl dimethyl benzyl ammonium chloride
lauryl dimethyl benzyl ammonium chloride (compound A)
lauryl dimethyl benzyl ammonium chloride (compound B)

The results obtained were compared with those obtained with sodium hypochlorite.*

*Sodium hypochlorite 3.25 percent, alkaline sodium phosphate 91.75 percent, and sodium chloride under 5.00 percent.

Technique — The bacteria-free broth filtrates were prepared by inoculating 200 ml. of protolysate (Mead-Johnson) vitamin fortified broth with two ml. of inoculum prepared by growing *S. lactis* culture H_6 on protolysate agar slants and adding 1.5 ml. of distilled water to each slant. Two slants were prepared for each 200 ml. of broth and incubated for 24 hours at 30°C. After an initial incubation period of six hours, an excellent growth of new host cells were obtained. Two ml. of bacteria-free filtrate containing the specific bacteriophage of a 10^{-13} titer were added and the flask re-incubated for three hours at 30°C. At the termination of the three hour secondary incubation period the broth had entirely cleared. This broth was filtered through a Selas 03 filter and pipetted into sterile tubes in eight ml. quantities.

For each of the compounds tested 168 tubes of bacteria-free filtrate were needed. These tubes were tested at time intervals of five, ten, and fifteen minutes and in dilutions of 1:100, 1:250, 1:500, 1:750, 1:1,000, 1:10,000, and 1:25,000. The broth filtrates were buffered with Prideaux and Ward's buffer to pH levels of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0.

One ml. of the quaternary ammonium compound being tested was added to each of three tubes of bacteria-free filtrate in each dilution and at each of the pH levels. At the proper time intervals, five, ten, and fifteen minutes, the effect of the quaternary ammonium compound was neutralized with one ml. of the proper dilution of sodium oleate. One ml. was pipetted from each treated tube and used in ascertaining the titer of the phage. The titer tests were made in litmus milk and read after incubation overnight at 30°C. The results were charted and compared with the results obtained from sodium hypochlorite.

The sodium hypochlorite compound was tested, with two exceptions, in the same manner. First, dillutions of five, ten, forty, and eighty-five parts per million of available chlorine were used. Second, available chlorine was neutralized with sodium thiosulfate.

Available chlorine was ascertained by titrating 50 ml. of the chlorine solution with N/10 sodium thiosulfate in the presence of two grams of potassium iodide and 10 ml. of 99.5 percent acetic acid, using starch solution as the indicator. The factor 70.9 times the number of ml. sodium thiosulfate used in the titration gave the number of parts per million in the 50 ml. of chlorine solution titrated. Starch solution and chlorine solutions were made daily.

Results — Quaternary ammonium compounds tested demonstrated some ability to inhibit the test strains of *S. lactis* bacteriophage. However, these compounds had a much lower inhibitive action than did sodium hypochlorite. Much higher dilutions of the quaternary ammonium compounds, containing 10 percent active ingredients, were needed to obtain an inhibitive action comparable to the commercial compound containing three and one-half percent sodium hypochlorite.

Experimentation on quaternary ammonium compounds indicated that pH was an important factor in phage inhibition. In the lower pH levels phage action was not as apparant as in higher pH levvels.

Although a slight inhibitive action was apparent at the different time intervals used, the difference was slight.

The clearing of the broth medium, in from two and one-half to three hours after inoculation with phage, indicated that the maximum phaging action was obtained during this interval.

Conclusion — Because quaternary ammonium compounds would come in contact with great amounts of protein, when employed as bactericidal agents in the cheese manufacturing industry, their use as inhibitive agents for phage is doubtful.

VITA

Howard E. Moorman was born at Christopher, Illinois on March 14, 1912. He attended both elementary and secondary schools in this city and in June 1936 received the degree of Bachelor of Education from Southern Illinois University.

From 1936 to 1943 he taught science in the high schools at Valier and Murphysboro, Illinois and during this interm completed his Masters degree in Bacteriology at the University of Illinois in 1942.

After spending three years with the 65th Infantry Division the author re-entered the University of Illinois in the fall of 1946 to devote full time to graduate study.

The author has held supervisory positions with the housing division and at the present is employed as a half time research assistant in the Chemistry Department.

He has at this time one publication, published in conjunction with Dr. G. I. Wallace of the Department of Bacteriology. Wallace, G. I., and Moorman, H. E. (1943), Bacteriology of Feet and Incidence of Dermatophytosis of Feet of High School Students. Archives of Dermatology and Syphilology June, Vol. 47: 816-821.